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# LETTER TO THE EDITOR

## Human Papillomavirus Genotype Prevalence in High-Grade Squamous Intraepithelial Lesions and Colposcopically Normal Women From Zimbabwe

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*Dear Sir,*

The paradigm shift that has occurred in the last decade attributing most, if not all, cervical cancers to infection with human papillomavirus (HPV) has renewed efforts to control cervical cancer, especially in developing countries where it remains the leading cause of cancer deaths among women. Primary and secondary prevention efforts have begun to focus on detection and control of the virus, specifically HPV DNA testing for screening[1–7] and HPV vaccine development for prevention.[8] Critical to the success of HPV-based prevention efforts is a comprehensive spectrum of targeted genotypes, given that at least 10 different HPV types have been classified as group 1 human carcinogens.[9] The International Biological Study of Invasive Cervical Cancer (IBSCC) demonstrated that certain HPV genotypes, namely, HPV-16, -18, -31 and -45, accounted for 80% of the sampled invasive cancers from 21 countries.[10,11] Based on these results, vaccine efforts are targeted first to HPV-16, with the hope of reducing the cervical cancer burden by up to 50%, presumably with vaccines targeting HPV-18, -31 and -45 to follow. However, a study in Mozambique found that HPV-35 was the most prevalent genotype, both in all HPV-positive women (16.7 %) and among women with cervical neoplasia (18.4%).[12] It is important to determine if this is a geographically isolated finding or if the relative prevalence of HPV types attributable to cervical cancer development differs in sub-Saharan Africa, where primary prevention offers the greatest promise of control. We report the genotype distribution of HPV from a nested case-control study of women originally enrolled in a visual inspection with acetic acid (VIA) screening study in Harare, Zimbabwe.[13]

Study participants were drawn from subjects enrolled in phase II VIA screening study conducted jointly by the University of Zimbabwe in Harare and the JHPIEGO Corporation, a Johns Hopkins University affiliate based in Baltimore, MD, USA. Details of subject recruitment have been described elsewhere.[13] Briefly, subjects enrolled in phase II of the VIA screening study were recruited from October 1996 through August 1997 among women aged 25–55 years attending 15 primary-care clinics in Chitungwiza and the greater Harare area of Zimbabwe. All enrolled women provided verbal informed consent, and the institutional review boards of both participating institutions approved study protocols. Participants were interviewed using a standardized questionnaire to assess demographics. Following the interview, each participant consented to a pelvic exam with collection of cells for Pap smear and HPV DNA testing. VIA screening was performed last. All participants were offered a colposcopic examination of the cervix, and biopsies were collected if indicated, usually on the same day. At the colposcopy visit, consenting women (23%) provided an oral mucosal specimen (OraSure; Epitope, Beaverton, OR) for HIV antibody testing (duplicate testing *via* commercially available ELISA; Organon Teknica, Durham, NC). Women consenting to an HIV test were slightly older, less likely to be married and likely to have more lifetime sexual partners than nonconsenting women.[6]

Colposcopic/histologic diagnosis was used for case identification as previously described.[5,6] A total of 215 cases with a final diagnosis of high-grade squamous intraepithelial lesion (HSIL) were identified. An equal number of potential controls of similar age were selected randomly from the pool of colposcopically normal participants; from these, 213 cervical swabs were available for HPV analysis. This resulted in a total sample size of 215 HSIL cases and 213 colposcopically normal controls.

All women participating in phase II with cells collected for HPV testing were screened for the presence of 13 cancer-associated HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68) using the HC2 (Digene Diagnostics, Gaithersburg, MD) microtiter plate test (probe B only).[5] A positive result was defined as any specimen whose relative light unit (RLU) was $\geq$ the 1.0 pg/ml reference RLU. Increasing specimen and reference RLU values were interpreted as a semiquantitative increase in viral burden.

Of the 600 μl sample collected for HPV DNA testing, 50 μl were removed for PCR analysis prior to HC2 testing. Each sample was denatured with a sodium hydroxide-based denaturing solution (Digene, Silver Springs, MD) equilibrated to

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one-half sample volume and mixed by vortex. Samples were then incubated in a dry heat block at 65°C for 45 min.

Following digestion, samples were centrifuged at 13,000 rpm for 15 min and the supernatant was precipitated overnight at −20°C using 100 μg glycogen, one-tenth volume of 5.0 M ammonium acetate and 2 volumes of 100% ethanol. The precipitation solution was centrifuged for 30 min at 13,000 rpm, the supernatant was removed and the pellet was washed with 70% ethanol and dried at room temperature for 1 hr. The dried DNA pellet was resuspended in 100 μl TE (pH 8.0) and stored at −80°C.

Sample DNA was amplified using the MY09/11/HMB01 L1 consensus primer system with coamplification of β-globin to assess specimen adequacy, as previously described.[14] The consensus PCR amplification targets a broad range of anogenital genotypes, which were subsequently discriminated using a reverse line blot hybridization method[15] targeting HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 58, 56, 58, 59, 68, 73 82, 83, 6, 11, 40, 42, 53, 54, 57, 66 and 84.

Pearson's $\chi^2$ test was used to assess case–control differences in distribution of binary exposures. For split-sample comparisons of PCR and HC2 HPV data, κ statistics were calculated to measure the percent agreement beyond that expected by chance, and McNemar's $\chi^2$ statistic tested for unequal distribution among the discordant results. Student's *t*-test was used to test for differences in mean number of viral types among HPV-positive women and differences in mean viral load by HCII. All statistical analyses were performed using Stata (College Station, TX) Version 7.0.

The 215 case and 213 control samples were tested for PCR adequacy using the β-globin control primers. Five case samples were excluded from the final analysis because they failed to produce a positive β-globin result. The final analytic sample, therefore, included 210 cases and 213 controls. Mean ages of the case and control groups were 31.8 and 31.9 years, respectively, indicating that control selection successfully represented the age distribution of the case group.

The overall HPV prevalence by L1 consensus PCR was higher in cases (77.1%) than controls (31.0%, $p < 0.001$), as expected (Table I). The high-risk (HR)-HPV prevalence by HC2 was 81.0% in cases and 32.9% in controls ($p < 0.001$), which was slightly higher in both groups relative to PCR. The absolute agreement between HCII and PCR was higher among the case group (91.4% agreement, $\kappa = 0.74$) than the control group (86.4% agreement, $\kappa = 0.69$). As evidenced by the higher prevalence by HC2 in both groups, there were significantly more $HC2^+/PCR^-$ samples among the discrepant results (McNemar's $\chi^2 = 4.8$, $p = 0.03$). The HPV prevalence by PCR decreased further to 73.3% among cases and 23.9% among controls when the $PCR^+$ results were restricted to samples positive for 1 or more of the 13 HR genotypes included in the HC2 probe pool. The agreement between PCR and HC2 should improve when restricting the comparison to include agreement to genotypes detectable by both methods. However, the agreement between HC2 and the restricted PCR results decreased to 89.5% among cases ($\kappa = 0.71$) and 85.9% among controls ($\kappa = 0.66$).

The genotype results for the case and control groups are shown in Table II. Of the 64 $HPV^+$ control women, a total of 124 infections were identified, representing 30 women with single infections and 34 women with multiple infections. HPVs 52, 51, 16 and 18 were the most commonly detected genotypes among the controls, representing 11.3%, 8.9%, 8.1% and 8.1% of the total infections, respectively. Of the 162 $HPV^+$ cases, a total of 523 infections were identified, with fewer women having single infections (52/162, 32.1%) than multiple infections (110/162, 67.9%). HPVs 16, 58, 18 and 52 were the most commonly detected genotypes among the cases, representing 12.2%, 8.8%, 8.2% and 7.6% of the total infections, respectively. In addition to having a higher prevalence of multiple infections relative to the control women, cases had a higher mean number of types per multiple infection (3.2 *vs.* 1.9 per sample, respectively; $p = 0.0001$).

A total of 99 of the 428 women selected for this study consented to HIV testing, representing 57 cases (27.0%) and 42 controls (19.7%). Results from 3 cases and 1 control were inconclusive for HIV status. Among the remaining 95 women with valid test results, HIV prevalence was 56.1% among controls and 77.8% among cases ($p = 0.02$). The HPV prevalence differed by HIV status, with 40.0% of $HIV^-$ women having a positive HPV result compared to 78.5% of $HIV^+$ women ($p < 0.001$). $HIV^+$ women also had a higher proportion of multiple infections (74.5 *vs.* 41.7%, $p = 0.03$) and more types present per infection relative to $HIV^-$ women (median 3 *vs.* 1 type, $p = 0.2$).

Most of the 27 genital HPV genotypes examined were detected at least once in this sample of women with HSIL and colposcopically normal women of similar age from Harare, Zimbabwe (HPV-42 excepted). Most remarkable in this analysis was the high proportion of multiple infections, especially among cases. While HPV-16 was the most frequently detected genotype among women with HSIL, it was part of a coinfection in 75% of cases. With such a high rate of multiple infections, it is difficult to estimate the attributable fraction of neoplasia for each genotype from such a population. However, given that each high-risk genotype is thought to have the potential to cause neoplasia, it must be assumed that all of the $HR\text{-}HPV^+$ lesions present in a multiple infection carry the potential for malignant progression. Certain genotypes, most notably HPVs 16, 18 and 52, predominated in both cases and controls. In a study from Costa Rica, no particular HPV genotype was found to predominate among cytologically normal women.[16]

Another study examining the prevalence of HPV genotypes in neighboring Mozambique found a predominance of HPV-35 among both cytologically normal women and women with neoplasia.[12] While we found an increase in HPV-35 prevalence

**TABLE I** – HPV DETECTION BY LINE BLOT PCR AND HYBRID CAPTURE 2

| | Number | PCR/HC2 result | | | | Agreement among positives | κ |
|---|---|---|---|---|---|---|---|
| | | +/+ | −/− | −/+ | +/− | | |
| Controls | 213 | 55 | 132 | 15 | 11 | 67.9% | 0.69 |
| HSIL | 210 | 157 | 35 | 13 | 5 | 89.7% | 0.74 |

**TABLE II** – GENOTYPE DISTRIBUTION AMONG WOMEN WITH HSIL *VS.* NORMAL COLPOSCOPY

| HPV genotype | Controls ($n$ = 213) | | Cases ($n$ = 210) | |
|---|---|---|---|---|
| | Number | % total infection | Number | % total infections |
| HR-HPV | | | | |
| 16 | 10 | 8.1 | 64 | 12.2 |
| 18 | 10 | 8.1 | 43 | 8.2 |
| 31 | 1 | 0.8 | 27 | 5.2 |
| 33 | 7 | 5.6 | 31 | 5.9 |
| 35 | 1 | 0.8 | 21 | 4.0 |
| 39 | 4 | 3.2 | 11 | 2.1 |
| 45 | 3 | 2.4 | 12 | 2.3 |
| 51 | 11 | 8.9 | 24 | 4.6 |
| 52 | 14 | 11.3 | 40 | 7.6 |
| 56 | 7 | 5.6 | 18 | 3.4 |
| 58 | 8 | 6.5 | 46 | 8.8 |
| 59 | 0 | 0 | 11 | 2.1 |
| 68 | 4 | 3.2 | 14 | 2.7 |
| Other HPV | | | | |
| 26 | 0 | 0 | 3 | 0.6 |
| 55 | 3 | 2.4 | 1 | 0.2 |
| 73 | 3 | 2.4 | 8 | 1.5 |
| 82 | 2 | 1.6 | 21 | 4.0 |
| 83 | 5 | 4.0 | 22 | 4.2 |
| LR-HPV | | | | |
| 6 | 5 | 4.0 | 13 | 2.5 |
| 11 | 2 | 1.6 | 6 | 1.1 |
| 40 | 0 | 0 | 1 | 0.2 |
| 42 | 0 | 0 | 0 | 0 |
| 53 | 9 | 7.3 | 34 | 6.5 |
| 54 | 5 | 4.0 | 17 | 3.3 |
| 57 | 1 | 0.8 | 0 | 0 |
| 66 | 6 | 4.8 | 14 | 2.7 |
| 84 | 3 | 2.4 | 21 | 4.0 |
| Total infections | 124 | 100% | 523 | 100% |
| Single infections | | 30/64 46.9% | | 52/162 32.1% |
| Multiple infections | | 34/64 53.1% | | 110/162 67.9% |
| 2 types per sample | | 19/64 (29.7%) | | 29/162 (17.9%) |
| 3 | | 9/64 (14.1%) | | 20/162 (12.3%) |
| 4 | | 2/64 (3.1%) | | 20/162 (12.3%) |
| 5 | | 3/64 (4.7%) | | 13/162 (8.0%) |
| 6 | | 1/64 (1.6%) | | 13/162 (8.0%) |
| 7 | | 0 | | 6/162 (3.7%) |
| 8 | | 0 | | 4/162 (2.5%) |
| 10 | | 0 | | 3/162 (1.9%) |
| 11 | | 0 | | 1/162 (0.6%) |
| 15 | | 0 | | 1/162 (0.6%) |

among cases relative to the African prevalence in the IBSCC study of invasive cancers (4.0% *vs.* 2.2%),[10] we did not see a predominance of HPV-35 relative to other genotypes. However, as Castellsague *et al.*[12] pointed out, the unexpected proportion of HPV-35 seen in Mozambique may have been attributable to utilization of a PCR test that has a higher sensitivity for certain types, including HPV-35, relative to the MY09/11 primer pair used in the present study. Indeed, the Mozambique study observed a higher agreement between HPV detection by HC2 and consensus PCR using PGMY/line blot (95.0%, $\kappa$ = 0.89), with PCR having a somewhat greater sensitivity overall, in contrast to the results presented here, which show HC2 having a higher overall analytic sensitivity. The genotype-specific lack of sensitivity shown to be true of MY09/11[17] may explain much of the HC2/PCR discrepancy seen in the present study, particularly since we see more similar agreement to the Mozambique study when restricting the comparison to the case group, which had a significantly higher average viral load,[5] and the high prevalence of HPV genotypes known to be amplified with poor efficiency using MY09/11 (*e.g.,* HPVs 35, 39 and 45).[17]

It is possible, therefore, that genotype-specific HPV prevalence may be underestimated depending on the assay used. Due to the high prevalence of multiple infections in this population, this type of misclassification is unlikely to significantly miss true HPV-infected individuals (since they are likely to have at least 1 HPV-type infection that is picked up by MY09/11 PCR) but may significantly underestimate the true genotype-specific prevalence in this population. This may be very important when planning for intervention strategies, such as vaccination, in such populations, as pointed out by Castellsague *et al.*[12]

HIV infection appears to be significantly associated with an increased prevalence of HPV infection in the study population, as well as the prevalence of multiple simultaneous infections. Because few women consented to HIV testing, however, we cannot exclude volunteer bias as a possible explanation for these differences. As noted in our full report of HPV screening in the context of an HIV-endemic population,[6] the women consenting to HIV testing in this study were at a generally higher risk for sexually transmitted infections relative to nonconsenters. This coupled with the periurban clinic recruitment strategy makes these results difficult to generalize to the entire population of Zimbabwe. However, our data appear to be consistent with many other populations of HIV-infected women and men, where a significant increase in anogenital

HPV prevalence and multiple infections has been reported.[18] The fact that genotypes other than HPV-16 are seen at relatively high frequency in this population may also be due to HIV, as others have reported that these HPV types, but not HPV-16, are more likely to increase in prevalence following HIV seroconversion (R. Burk, personal communication).

Together, these data are important to the design of rational vaccine strategies in the sub-Saharan African nations. Cancer-associated HPV genotypes not currently considered in type-specific vaccine formulations are highly prevalent in these countries. The actual prevalence of some types may be even greater, given that these types, which are more rare in the developed countries, have not been adequately detected by the commonly used DNA detection methods. Identification of HPV genotypes in lesion tissue by *in situ* hybridization may be useful to determine which of the multiple types isolated from a specimen was responsible for the lesion. Furthermore, the effect of HIV-induced immunosuppression on cervical cancer incidence in this population is unknown. It is clear that HIV-induced immunosuppression leads to inadequate clearance of HPV infections.[19] It is therefore of great concern that untreated HIV infection may compromise the efficacy of preventive vaccination in this and other HIV-endemic regions. To this end, it is important to begin planning HPV trials that will include $HIV^+$ women.

*Yours sincerely,*

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